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<p>(21) International Application Number: PCT/GB94/00694 (22) International Filing Date: 31 March 1994 (31.03.94) (30) Priority Data: 9307250.2 7 April 1993 (07.04.93) GB 9402573.1 10 February 1994 (10.02.94) GB (71) Applicant (for all designated States except US): THE INSTITUTE OF CANCER RESEARCH: ROYAL CANCER HOSPITAL [GB/GB]; 17A Onslow Gardens, London SW7 3AL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): MARSHALL, Christopher, John [GB/GB]; 1 Highcroft, 30 Old Lodge Lane, Purley, Surrey CR8 4DF (GB). ASHWORTH, Alan [GB/GB]; 88 Drayton Gardens, London SW10 9RF (GB). HUGHES, David, Anthony [GB/GB]; 67A Cromford Road, Wandsworth, London SW18 1PA (GB). (74) Agents: ARMITAGE, Ian, Michael et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p>	<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: METHODS FOR SCREENING OF SUBSTANCES FOR THERAPEUTIC ACTIVITY AND YEAST FOR USE THEREIN</p> <p>(57) Abstract</p> <p>Methods of screening for substances which affect mammalian MAP kinase pathways, both inhibitors and activators, are provided. Substances identified using the methods as having such an effect are candidate pharmaceuticals for use in treatment of cancer, inflammatory disorders, cardio-vascular disorders or neurological disease. Yeasts are provided for use in the methods. In the yeast, deficiencies in yeast MAPKK kinase and MAPK kinase are complemented by mammalian MAPKK kinase and MAPK kinase. Yeast MAPK may also be replaced with a mammalian homologue and mammalian MAPK phosphatases may be introduced.</p>		

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METHODS FOR SCREENING OF SUBSTANCES
FOR THERAPEUTIC ACTIVITY AND YEAST FOR USE THEREIN

5 This invention relates to the screening of
candidate substances for potential as pharmaceutical
agents. More particularly, it provides a method by
which test substances can be screened for their
ability to affect a MAP kinase pathway in mammals.
10 Methods are provided for screening test substances
for inhibition or activation of the pathway. The
invention also provides yeast which are of use in
the methods.

 It is well known that pharmaceutical research
leading to the identification of a new drug
15 generally involves the screening of very large
numbers of candidate substances, both before, and
even after, a lead compound has been found. This is
one factor which makes pharmaceutical research very
expensive and time-consuming, so that a method for
20 assisting in the screening process can have
considerable commercial importance and utility.

 In mammalian cells the activation of the enzyme
MAP kinase (MAPK) is a consequence of growth factor
stimulation, and is a requirement for cell
25 proliferation (61). Since oncogenic p21 ras
proteins transform cells, and inhibition of the
normal p21 ras proteins in cells interferes with
growth factor signalling, it has been generally
assumed that these proteins are involved in the
30 control of cell proliferation. In particular it
appears that they are involved in transmitting
signals from growth factor receptors to cytoplasmic
signal transduction pathways, since both tyrosine
kinase-type growth factor receptors and non-tyrosine
35 kinase growth factor receptors require normal p21
ras functions to stimulate MAPK activity and cell
proliferation. It seems, therefore, that oncogenic

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forms of p21 ras uncouple the activation of MAPK from the requirement for external growth factor signals.

5 It has also been found that the activation of intracellular protein kinase C (PKC) by phorbol esters stimulates MAPK activity without normal ras function in some cell types. It has further been shown that oncogenic p21 Ras introduced into quiescent 3T3 cells rapidly activates PKC and leads to the activation of MAPK in the absence of any external stimuli.

10 It seemed to us that the activation of MAPK from ras or PKC proceeds successively via the Raf protein kinase and MAPK kinase (MAPKK), essentially along the lines:

	TK		PKC	
	v		v	
20	Ras	----->	Raf (MAPKKK)	--> MAPKK --> MAPK

It should be noted that there is a family of MAP-kinases and that the pathway is implicated in many diverse cell types [35-37]. Two forms of MAP kinase have been purified from fibroblasts with molecular weights P42^{mapk} and P44^{mapk}, (ERK-2 and -1 respectively), [38]. Activation requires an ordered phosphorylation of a threonine and tyrosine located within the conserved kinase subdomain 8, (T183, Y185), [39,40].

30 Yeast MAPK-pathway homologue proteins are involved in yeast signal transduction, including in response to mating pheromones. In the case of the yeast *Schizosaccharomyces pombe* one MAPK protein is Spk1. Two additional kinases, Byr1 and Byr2, lie in the same pathway as Spk1, of which Byr1 has been shown to have some sequence homology to MAPKK. In

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addition, the mating pheromone pathway in Spk1 requires Ras protein function, and Byr1 and Byr2 are thought to act downstream of Ras in this pathway. It is possible, therefore that the way in which ras is coupled to these kinase cascades is similar in fission yeast and higher eukaryotes. More particularly, we believe a pathway in *S. pombe* to be essentially of the form:

10 Ras --> Byr2 --> Byr1 --> Spk1

There are equivalent proteins in as follows:

	<i>S. pombe</i>	<i>S. cerevisiae</i>
15	Byr1 (also known as STE1)	= STE7
	Byr2 (also known as STE8)	= STE11
	Spk1	= FUS3/KSS1

20 Additionally, in *Saccharomyces cerevisiae* there are other pathways with MAP kinase homologues and components with equivalent function to those in the mammalian MAPK pathway: the HOG1 and MPK1 pathways. MPK1 is a yeast MAPK and has the following components in its pathway:

PKC1 BCK1 (= MAPKKK) MKK1/MKK2 (= MAPKK) MPK1
(= MAPK);

30 The yeast MAP kinase HOG1 has the following components in its pathway:

PBS2 (= MAPKK) HOG1 (= MAPK) (53)

35 In all cases, various additional components may act upstream in response to a stimulus, which may come from outside the organism.

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Surprisingly, when we placed a mammalian (human) Raf, or a deletional derivative thereof, together with MAPKK, in a yeast strain deficient in either byr1 or byr2, the engineered strain would
5 mate, indicating that the pathway was functioning, while expression of raf or the raf derivative alone or MAPKK alone did not allow byr1 or byr2 mutant cells to mate. This strongly suggests that Raf can directly phosphorylate and activate MAPKK. It also
10 suggested to us the replacement of spk1 with MAPK and/or yeast ras with mammalian (human) ras in yeast.

From a practical viewpoint, this experiment reconstructed part of mammalian MAPK pathway in an
15 organism which is amenable for use in screening, eg for inhibitors of this pathway.

We have also shown that the Mos protein kinase can activate MAPKK expressed in yeast. The *c-mos* gene was first identified as the cellular homologue
20 of a transforming gene (*v-mos*) from a mouse retrovirus (54), and it was subsequently shown that *c-mos* can also transform mammalian cells. The *c-mos* gene product (Mos) is a serine/threonine kinase expressed in germ cells. Extensive studies have
25 been done on Mos in *Xenopus* (frog) where was shown to be necessary for meiotic maturation of oocytes in response to progesterone.

Although Mos expression is generally confined to germ cells, it is possible that inappropriate
30 expression of Mos could lead to oncogenesis through activation of the MAP kinase pathway. Indeed high level expression of Mos protein has been detected in cervical carcinoma-derived cell lines (Li et al., 1993). Inhibitors of Mos kinase could have
35 therapeutic potential in tumours that express Mos.

Two published studies have suggested that Mos is involved in the activation of *Xenopus* MAPK during

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meiosis (56, 57). Furthermore, a bacterially expressed maltose-binding protein (MBP)-Mos fusion protein could activate purified, phosphatase-inactivated MAPKK, suggesting that Mos could be a MAPKKK (56). However, the MBP-Mos fusion protein had to be "activated" by incubation in a cell extract (rabbit reticulocyte lysate) so that it was difficult to eliminate the possibility that a MAPKKK in the reticulocyte lysate that associates with MBP-Mos was responsible for the *in vitro* activation of MAPKK, and not Mos itself.

The work described in Example 2 confirmed that Mos works in yeast in a manner similar to Raf-1, directly phosphorylating MAPKK, and so can be termed a MAPKK kinase or MAPKKK.

The full picture of how the MAP kinase pathway is switched off is as yet unclear. Down-regulation of MAP kinase activity by de-phosphorylation is likely to be of key importance. The human gene CL100 [41] and its murine homologue 3CH134 [42] were originally discovered as genes whose transcription was stimulated by growth factors, oxidative stress and heat shock. Subsequently, they were shown to encode polypeptides that have both serine/threonine and tyrosine phosphatase activity [43-44]. This removal of phosphate from both threonine and tyrosine on MAP kinase is unusual. When expressed *in vitro* [43-44] this gene product has been shown to be very specific for MAP kinase and leads to its inactivation. Co-expression of the murine gene 3CH134 and the erk2 MAP kinase isoform in mammalian cells leads to the dephosphorylation and inactivation of the MAP kinase [45]

Disclosed herein are several new genes, each encoding a polypeptide implicated in the MAP kinase regulatory system.

Several nucleic acid molecules have been

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discovered and isolated encoding proteins which are related to the known MAP kinase phosphatases. Using insight gained from specialist knowledge in the field, an investigative procedure was designed which resulted in the obtention of the new genes. The actual procedure used is described in detail below, and disclosed, along with the phosphatases, in patent application GB 9402573.1.

The sequences of the polypeptides encoded by the novel nucleic acid sequences share a degree of homology with the sequence of the known MAP kinase phosphatase, CL100, which is sufficient for indication as phosphatases, particularly MAP kinase phosphatases.

MAP kinase phosphatases are likely to act as off switches for cell proliferation. The fact that there are multiple MAP kinase phosphatases suggests that there may be some specificity to the off switches. Activators of the MAP kinase phosphatases, either general or for specific family members, may be anti-proliferative agents. Provision of nucleic acid encoding phosphatases enables screening for such activators. Loss of MAP kinase phosphatase activity by, for example, mutation may lead to uncontrolled cell proliferation. Hence, some of these genes may prove to be tumour suppressor genes.

According to a first aspect of the present invention there is provided a method of screening for a substance which is an inhibitor of mammalian MAPK pathway, which comprises:

taking yeast which is deficient for yeast MAPKK kinase and MAPKK gene activity, and wherein the deficiency is complemented by coexpression of mammalian MAPKK kinase and MAPKK genes;

exposing the yeast to a test substance under conditions which would normally lead to the

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activation of the yeast MAPK pathway; and
looking for an end point indicative of
activation of the yeast MAPK pathway;
whereby inhibition of that endpoint indicates
5 inhibition of the MAPK pathway by the test
substance.

According to a second aspect of the present
invention there is provided a method of screening
for a substance which is an inhibitor of mammalian
10 MAPK phosphatase action on MAPK, which comprises:
taking a yeast which is deficient for MAPKK
kinase and/or MAPKK gene activity, wherein the
deficiency is complemented by coexpression of
mammalian MAPKK kinase and MAPKK genes and wherein a
15 mammalian MAPK phosphatase gene is expressible;
exposing the yeast to a test substance under
conditions wherein the MAPK phosphatase normally
inhibits the yeast MAPK pathway; and looking for an
end point indicative of activation of the yeast MAPK
20 pathway;
whereby activation of that endpoint indicates
inhibition of MAPK phosphatase action on the MAPK by
the test substance.

According to a third aspect of the present
25 invention there is provided a method of screening
for a substance which affects mammalian MAPK
phosphatase action on mammalian MAPK pathway which
comprises:
taking a yeast which is deficient for MAPKK
30 kinase and/or MAPK gene activity, wherein the
deficiency is complemented by coexpression of
mammalian MAPKK kinase and MAPKK genes and wherein a
mammalian MAPK phosphatase gene is expressible;
exposing the yeast to a test substance under
35 conditions wherein the MAPK phosphatase is expressed
and normally partially inhibits the yeast MAPK
pathway; and looking for an end point indicative of

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activation or further inhibition of the yeast MAPK pathway;

whereby activation of that endpoint indicates inhibition of MAPK phosphatase action by the test substance, and further inhibition of that endpoint indicates either activation of MAPK phosphatase action by the test substance or inhibition of the MAPK pathway by the test substance.

The yeast may be any strain of *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, (eg *Saccharomyces carlsbergensis*), or *Candida albicans*, though the asexual nature of this last yeast, and the fact that it is diploid, make mutation and selection more difficult. A MAPK homologue has been stored from *Candida albicans* (58). In *Schizosaccharomyces pombe* the MAPKK kinase and MAPKK may be Byr1 and Byr2 respectively. In *Saccharomyces cerevisiae* they may be STE7 and STE11 in the FUS3/KSS1 pathway or equivalents in other MAPK pathways, as discussed supra.

Neiman et al (52) demonstrated interchangeability of *S. pombe* genes *byr2*, *byr1* and *spk1* with *S. cerevisiae* genes STE11, STE7 and FUS3. Mutations in one species can be complemented by expression of the equivalent genes from the other, illustrating the conservation of function of the kinases between the species.

The yeast MAP kinase gene (eg *spk1*) may be replaced by a mammalian MAPK gene able to function in the yeast environment. This may be particularly desirable when substances are to be tested for effect on MAP kinase phosphatase action of MAPK pathway. Neiman et al (52) demonstrated that the mammalian MAP kinase ERK2 can function in place of *spk1* in *S. pombe*. Likewise, Gotoh et al (19) demonstrated that *Xenopus* MAPK can act in *S. pombe* in place of *spk1*. Yeast components upstream of

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MAPKK kinase, eg Ras, may also be replaced by a mammalian homologue.

The end point of the screen may be the mating ability of the yeast or the ability to sporulate, or it can be an artificially constructed end point obtained by making an activated component such as Spk1 or MAPK switch on a reporter gene, in known manner. For instance, a reporter for Spk1 activation may be the promoter of a gene that is regulated by the ras-spk1 pathway in response to mating pheromones, such as *matPm* (65) or *sxa2* (66), fused to a reporter gene such as *lacZ* encoding β -galactosidase. A suitable reporter system for mammalian MAPK activation may be based on phosphorylation by MAPK activating a GAL4-Elk-1 fusion protein, which acts as a transcription factor to stimulate expression from a GAL4 operator. If the GAL4 operator is fused to a reporter gene, such as *lacZ*, and incorporated into the yeast, there will be a detectable end-point.

The reporter gene is likely to encode an enzyme which catalyses a reaction which produces a visually detectable signal, such as a coloured product. Many examples are known, including β -galactosidase and luciferase. β -galactosidase activity can be assayed by production of blue colour on substrate, the assay being visual or by use of a spectrophotometer to measure absorbance. Fluorescence, eg that produced as a result of luciferase activity, can be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. Spontaneous fluorescence, such as that of green fluorescent protein disclosed by Chalfie et al (60), may be used. The product of activity of a reporter gene may be assayed, to determine gene activity, using a

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specific binding pair member able to bind the product, eg. an antibody.

The MAPKK kinase may be Raf, Mos, MEK kinase (Lange-Carter et al 1993) or any other mammalian protein which can activate MAPKK by phosphorylation.

Variants, mutants or derivatives of a wild-type MAPKK kinase, (eg raf or mos) MAPKK, MAPK or MAPK phosphatase gene may be used. Variants and mutants have some change to the wild-type nucleic acid sequence. The change may be one or more of insertion, deletion or substitution of one or more nucleotides resulting in either no change of amino acid sequence of the encoded protein or a change affecting one or more amino acid residues in the encoded protein, which may or may not affect the protein function. The methods of the present invention enable testing of mutants, variants or derivatives which are naturally occurring or created artificially in vitro. This is likely to broaden the range of useful activators or inhibitors of elements of the MAPK pathway, such as MAPK phosphatases, which can be found using the present invention.

Following identification of a substance which affects components of the pathway, an inhibitor of MAPK, an inhibitor or activator of MAPK phosphatase and so on, the substance may be manufactured or used, for instance in the preparation of a medicament. Such a medicament may particularly be for treatment of a proliferative disorder (eg cancer) in a mammal, or treatment of other disorders where MAP kinases may be implicated, such as inflammatory disorders (63), cardio-vascular disorders (64) and neurological disease (22) (Nerve Growth Factor activates a MAPK cascade.) The manufacture and/or use of a substance identified using the present invention fall within the scope of

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the invention.

Additionally, the present invention extends to a substance identified by a method according to the invention as an inhibitor of mammalian MAPK pathway, or as a substance which affects mammalian MAPK phosphatase action on MAPK (eg an activator or inhibitor of this action), for use as a pharmaceutical, and the use of such substances in the preparation of a medicament for the treatment of any one or more of a proliferative disorder, an inflammatory disorder, a cardio-vascular disorder and a neurological disorder.

According to another aspect of the present invention there is provided yeast which is defective in yeast MAPKK kinase and/or MAPKK gene activity, which defect is complemented by the coexpression of mammalian MAPKK kinase and MAPKK genes. The yeast may be *Schizosaccharomyces pombe* (*byr1* and/or *byr2* gene activity may be defective) *Saccharomyces cerevisiae* (in which case the defective genes may be *STE7* and/or *STE11*, or the equivalents in another MAPK pathway), or *Candida albicans*. (For further discussion of this see *supra*.) The yeast MAPK (eg Spk1) may also be replaced by a mammalian MAPK, and means for assessing MAPK activity designed accordingly (ie the end-point for the screening methods according to the invention). Upstream components of a subject pathway (eg Ras) may also be replaced with a mammalian homologue.

A number of mammalian MAPK pathways are known to exist. It may be that in a particular case a factor found in mammalian cells but not in yeast is required for activity of one of the components of a pathway e.g. MAPKKK, MAPKK, MAPK. Then, if that particular component is to be used in one of the screening methods of the invention, either the factor will have to be introduced into the yeast, eg

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by cloning the gene encoding the factor and introducing it into the yeast so that the factor is expressed, or by mutating the component in a way which removes its requirement for the factor. (Raf-
5 1, as an illustration, can be activated by deletion of an N-terminal domain.)

The yeast may further contain nucleic acid from which a mammalian MAPK phosphatase is expressible, to enable screening for substances which interfere
10 with the action of MAPK phosphatase on MAPK, mammalian or yeast, (Spk1, FUS3, KSS1, HOG1 or MPK1, etc). The MAPK phosphatase may be CL100, 3CH134 or any of the phosphatases made available herein. Sequence information is given in the figures. As
15 already discussed, the phosphatase may be a variant, mutant or derivative of the wild-type.

Preferably, the mammalian MAPK phosphatase is over-expressed, ie expressed at a level which is high enough to mask any effect of yeast phosphatases
20 on Spk1 or mammalian MAPK (if present in place of Spk1) in a screening method according to the invention. It may be desirable in certain circumstances to disrupt a yeast phosphatase gene function to stop or reduce any interfering action
25 the yeast phosphatase might otherwise have on screening for substances which affect mammalian MAPK phosphatase action. For instance, if the mammalian phosphatase is not over-expressed but is expressed at a relatively low level, it may be that endogenous
30 yeast phosphatase will act on the MAPK in the yeast to an extent that any effect (activation or inhibition) of the test substance on the mammalian phosphatase action on MAPK is not detectable.

Techniques for disrupting gene function are
35 known and facilitated by the fact that a *Saccharomyces cerevisiae* gene encoding a phosphatase which acts on FUS3/KSS1 has been cloned (59). A

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combination of in vitro mutagenesis and homologous recombination may be used to disrupt this gene's function. Furthermore, other phosphatase genes in yeast are likely to have sequences homologous to this gene and so may be cloned using primers or probes with sequences based on parts of the cloned gene, then mutated or disrupted in some way before being used to replace the wild-type gene in a yeast chromosome.

The yeast according to the present invention are useful in the methods described herein for the identification of useful substances.

The mammalian genes may be introduced into yeast on autonomously replicating plasmids and propagated as extrachromosomal elements as illustrated herein. These vector plasmids, known as shuttle-vectors, contain sequences for replication and selection both in bacteria and yeast [46]. Other controlling elements such as promoter sequences and transcription termination sequences are included for expression of the mammalian genes [47-48]. The controlling elements may be derived from yeast or from other organisms or viruses.

Alternatively the mammalian genes may be introduced into the yeast genome. This may be achieved by random, non-homologous recombination or by homologous recombination directed by cloned yeast sequences into a predetermined site in the chromosome [49]. Expression of the mammalian genes would be regulated by controlling elements like those used in plasmid vectors. Different promoter sequences may be used to vary the level of expression of the mammalian gene products [50].

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences,

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marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

5 The methods of the present invention will identify substances which interfere with the activity of a component in the pathway or which interfere with the interaction of two or more components which each other. The functioning of any enzymatic cascade depends on both enzymatic activity
10 of each component and the ability of each component to interact with another component.

 The experimental basis for the invention and illustrative embodiments of the invention will now
15 be described in more detail, with reference to the accompanying drawings. All publications mentioned in the text are incorporated herein by reference.

 Figure 1 shows complementation of the mating defect of a *byr1* mutant by coexpression of Raf and MAPKK. Micrographs of a *byr1* mutant transformed
20 with MAPKK alone (a), MAPKK plus *raf-1* (b), MAPKK plus Δ *raf-1* (c), *raf-1* alone (d), Δ *raf-1* alone (e), and *S. pombe byr1*⁺ (f).

 Figure 2 shows MAPKK activity in *S. pombe* cells coexpressing Raf. A; MAPKK and MAPK activities of
25 fractionated cell extracts from a *byr1* mutant strain (CB53) transformed with MAPKK and Δ *raf-1*. B; Immunoblot of column fractions to detect rabbit MAPKK.

30 Figure 3 shows stimulation of MAPKK phosphorylation by *raf* in *S. pombe*. A; Immunoprecipitation of rabbit MAPKK in cells expressing MAPKK alone (lane 1), MAPKK plus *raf-1* (lane 2), MAPKK plus Δ *raf-1* (lane 3), *raf-1* alone (lane 4) and Δ *raf-1* alone (lane 5). Top: phosphor
35 imager print showing phosphate-labelled MAPKK (lanes 1-3). Bottom: immunoblot of the same

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immunoprecipitated samples with the anti-MAPKK serum. B: Phosphopeptide maps of MAPKK. The origin is marked with a cross (bottom left of each panel). The horizontal dimension is electrophoresis (cathode to right) and the vertical dimension is chromatography.

Figure 4 shows complementation of the mating defect of *byr1* of *byr2* mutants by coexpression of mammalian MAPKK and Mos. (a) to (d), *byr1* mutant transformed with either MAPKK alone (a), MAPKK and Mos (b), Mos alone (c), or *byr1*+(d), (e) to (h), *byr2* mutant transformed with either MAPKK alone (e), MAPKK and Mos (f), Mos alone (g), or *byr2*+(h). METHODS. Mouse c-mos cDNA was cloned into pREP52, a derivative of pREP42 (Basi et al., 1993). The *byr2* mutant strain CB85 (*h⁹⁰byr2::ura4 Δ ARS ade6 leu1 ura4*) was derived from JX3 (Gotoh et al., 1993). The other plasmids and the *byr1* mutant strain CB53 have been described previously (Hughes et al., 1993). The transformants were photographed after 3 days on synthetic sporulation agar (SSA).

Figure 5 shows MAPK kinase activity in a *byr1* mutant expressing MAPKK and Mos. Cell extracts were prepared from a *byr1* mutant strain (CB53) expressing either MAPKK alone, MAPKK and Raf-1, or MAPKK and Mos 5 μ g of total protein from each extract was assayed for MAPKK activity as described previously (Hughes et al., 1993). Expression of each of the kinases in the extracts was confirmed by immunoblot analysis (data not shown).

Figure 6 shows DNA sequences of novel phosphatase molecules. STY2-STY4 are PCR products amplified from RNA produced from A431 cells as described in the text. STY6 is part of a cDNA clone isolated by screening a human liver cDNA library with a mixture of STY2 and STY3 probes shown in part a) and b). STY7-STY10 are parts of cDNA clones

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isolated by screening a human brain cDNA library with a mixture of STY2 and STY3 probes shown in part a) and b). All sequences apart from STY7 and STY10 show homology to CL100. In the case of these clones the sequence shown does not show homology to CL100 but the cDNA clones hybridised strongly to the STY2/3 probe suggesting that these clones also encode novel phosphatase genes. Figure 6 (a) shows STY2, Figure 6 (b) shows STY 3, Figure 6 (c) shows STY4, Figure 6 (d) shows STY 5, Figure 6 (e) shows STY6, Figure 6 (f) shows STY 7, Figure 6 (g) shows STY 8, Figure 6 (h) shows STY 9 and Figure 6 (i) shows STY10.

Figure 7 shows deduced amino acid sequences of phosphatase clones aligned with the amino acid sequence of CL100. For parts a)-c) spaces indicate residues that are identical with CL100 and dots indicate residues which have not yet been determined. For part d) which is a comparison of the full length clone for STY8 with CL100 dashes (-) indicate gaps introduced into the sequences to optimise their alignment. Shaded residues correspond to residues that are identical between STY8 and CL100.

The amino acid sequences shown correspond to residues 177-255 of STY2, STY3, STY4 and STY5 for Figure 7 (a), 231-302 of STY6 for Figure 7(b), 223-267 of STY9 for Figure 7 (c) and 1-367 of STY8 for Figure 7 (d).

Figure 8 shows proof that STY8 encodes MAP kinase phosphatase activity. Protein extracts were prepared from COS cells transfected with various recombinant plasmids before or after stimulation of the cells with EGF. These extracts were electrophoresed on SDS/polyacrylamide gels and the proteins then transferred to a nitrocellulose membrane. This membrane was then incubated with the

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anti-myc antibody 9E10, treated by the ECL procedure and the resulting chemiluminescence detected on x-ray film. It can be seen that in the absence of stimulatory ligand (EGF) the anti-myc antibody 9E10 reveals only a single band of MAP kinase on western blotting (lane 1). In the presence of EGF (lane 2) a clear doublet of bands is present indicating the partial phosphorylation of the MAP kinase. This is unaffected by expression of the parental expression vector (lanes 3 and 4). However, expression of CL100 or STY8 in the presence of EGF (lanes 7-10) leads to abolition of the EGF induced shift indicating that both these molecules encode MAP kinase phosphatases. Lanes 5 and 6 in which the cells are transfected with Myc-tagged STY8 shows that the STY8 protein is indeed expressed. Lane 1 is MAPK; Lane 2 is MAPK + EGF; Lane 3 is MAPK + pMT; Lane 4 is MAPK + pMT + EGF; Lane 5 is Myc - STY8; Lane 6 is Myc - STY8 + EGF; Lane 7 is MAPK + CL100; Lane 8 is MAPK + CL100 + EGF; Lane 9 is MAPK + STY8; Lane 10 is MAPK + STY8 + EGF.

EXAMPLE 1

Referring firstly to Fig. 1; rabbit MAPKK cDNA and *S. pombe* byr1 were cloned into pREP41 and human raf-1 and Δ raf-1 were cloned into pREP42. The raf-1 clone encodes the full length Raf-1 protein whereas in Δ raf-1 the first 324 amino acids are detected (7). The vector plasmids are derivatives of the nmt1 promoter plasmids²⁹ and carry either the *S. cerevisiae* LEU2 gene (pREP41) or the *S. pombe* ura4⁺ gene (pREP42) as selectable markers³⁰. A null mutant of byr1, with a 0.18-kilobase deletion (SpeI to BamHI) of the open reading frame, was constructed by one-step gene disruption. The byr1 mutant strain CB53 (h⁹⁰ byr1::ura4 Δ RA ade6 leu1 ura4) was transformed with two plasmids, one derived from

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pREP41 and the other from pREP42, and Leu⁺ Ura⁺ transformants carrying both plasmids were selected. Transformants were grown on synthetic sporulation agar (SSA)³¹ for 3 days and then photographed.

5 Molecular genetics methods for *S. pombe* were as described^{32,33}.

Referring to Fig. 2; cells were grown in minimal medium (EMM) to ca 1x10⁷ cells/ml, harvested by centrifugation and washed in stop buffer (150 mM NaCl, 50 mM NaF, 10mM EDTA, 1mM NaN₃, pH 8.0). Cells (2.0x10⁸) suspended in 20μl 50mM Tris. Cl pH 7.3, 40mM Na₂P₂O₇, 50mM NaF, 5mM MgCl₂, 0.3mM Na orthovanadate, 10mM EGTA, 1% Triton X-100, 20μg/ml leupeptin, 20μg/ml aprotinin, 1mM PMSF were broken
15 with 1g glass beads by vortexing for 2 min, the beads washed with 50 volumes of buffer A (50mM Tris.Cl pH 7.0, 2mM EDTA, 2mM EGTA, 0.1% β-mercaptoethanol, 5% glycerol, 0.03% Brij35, 0.3mM Na orthovanadate, 1mM benzamidine, 4μg/ml leupeptin)
20 and the lysate cleared by centrifugation in an Eppendorf microcentrifuge at 14,000 rpm for 10min. 0.5ml cleared lysate (1.5mg total protein) was then applied to a 1ml Mono-Q column (Pharmacia L.K.B.) and the column developed in buffer A with a 25ml
25 linear salt gradient to 0.35M NaCl. The flow rate was 1ml min⁻¹ and 0.5ml fractions were collected and assayed for myelin basic protein (MBP) kinase activity after preincubation with recombinant ERK2 (+ERK2) for MAPKK activity or after preincubation
30 with buffer (-ERK2) for MAPK activity, essentially as described by Traverse et al²⁴.

(B) Aliquots of each fraction assayed for kinase activity were resolved by 10% SDS-PAGE, electroblotted to Immobilon (Millipore) and probed
35 with rabbit polyclonal antibody 179 raised against a GST-rabbit MAPKK fusion protein (A.Ashworth and C.J. Marshall, unpublished) and ECL reagents (Amersham).

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Referring to Fig. 3; cells were grown in low-phosphate EMM to mid log phase (ca 5×10^6 cells/ml), labelled with [32 P] orthophosphate for 3.5h³² and then broken with glass beads in lysis buffer (25mM Tris.Cl pH 8.0, 40mM Na₂P₂O₇, 50mM NaF, 5mM MgCl₂, 0.1mM Na orthovanadate, 10mM EGTA, 1% Triton X-100, protease inhibitors: 20μg/ml aprotinin, 20μg/ml leupeptin, 1mM PMSF). After centrifugation (15,000 rpm for 15 mins) the supernatants were adjusted to 0.5% sodium deoxycholate (Na DOC), 0.1% sodium dodecyl sulphate (SDS), and incubated for 1 h with anti MAPKK serum 179 precoupled to protein A-sepharose. The beads were washed four times with buffer (lysis buffer with Na DOC and SDS, without MgCl₂ and protease inhibitors), incubated with 0.1 mg/ml RNase for 30 minutes, washed again, suspended in Laemmli's sample buffer and boiled. Samples were resolved by 10% SDS-PAGE and electroblotted to Immobilon. After autoradiography MAPK was detected using the anti-MAPKK serum 179 and alkaline-phosphatase-conjugated secondary antibody (Promega). Radioactivity was quantitated using a phosphorimager (Molecular Dynamics) and the immunoblot was scanned on a scanning densitometer (Joyce-Loebl). Two-dimensional phosphopeptide maps were obtained after trypsin digestion of MAPKK on Immobilon. The first dimension was electrophoresis in pH 1.9 buffer at 400 V for 60 min on cellulose thin-layer plates (Kodak); the second dimension was ascending chromatography developed with phosphochromatography buffer for 3 h³⁴.

DISCUSSION

Mammalian MAP kinase kinases (MAPKKs) are structurally related to the *byr1* gene product of the fission yeast *S. pombe*. Rabbit MAPKK, for example, is 55% identical in amino-acid sequence to Byr1 in

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the catalytic domain and 38% identical overall. To see whether mammalian MAPKK might be able to complement the byr1 mutant defect, a rabbit MAPKK cDNA driven by a fission yeast promoter was transformed into a byr1 mutant strain. Expression of MAPKK could not complement the mating defect of this strain (Fig. 1). Since MAPKK must be activated by phosphorylation²², it was possible that *S. pombe* did not have such an activator. The product of the *raf-1* protooncogene has been implicated in activation of the MAP kinase pathway in mammalian cells, perhaps as a direct activator of MAPKK⁶⁻⁸, so we examined whether Raf-1 could activate MAPKK in *S. pombe*. When byr1 mutant cells coexpressed either Raf-1 or Δ Raf-1, an activated derivative of Raf-1, together with MAPKK they were able to mate (Fig 1), although the mating frequency was lower than that of cells carrying the wild-type byr1 gene (Table 1). Expression of Raf-1 or Δ Raf-1 alone or MAPKK alone did not allow byr1 mutant cells to mate, showing that expression of both MAPKK and Raf is required to substitute for Byr1.

The *S. pombe* gene *spk1*, encodes a protein kinase thought to be involved in the same pathway as Byr1^{9,19}. The Spk1 kinase is homologous to vertebrate MAP kinases and to *S. cerevisiae* FUS3 and KSS1 and like them contains the regulatory TEY phosphorylation site motif in subdomain VIII⁹. Coincident with the work we describe here, others have shown that *Xenopus* and mammalian MAPK's can act in place of Spk1 in *S. pombe*¹⁹, (52). By analogy to other systems^{21,23} and from genetic and biochemical analysis¹⁹ it is probable that Byr1 phosphorylates and activates Spk1. Thus activated MAPKK may be substituting for Byr1 by phosphorylating and activating the Spk1 kinase in *S. pombe*. Consistent with this hypothesis, coexpression of MAPKK and Raf

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could not rescue the mating deficiency of a *spk1* null mutant (Table 1).

5 These experiments show that mammalian MAPKK can function in *S. pombe* with coexpression of Raf. To investigate whether the kinase activity of MAPKK was dependent on Raf, cell extracts were prepared from *S. pombe* cells expressing MAPKK alone, MAPKK plus Δ Raf-1 or Δ Raf-1 alone, fractionated on a Mono Q (trade mark) ion exchange column and the fractions
10 assayed for MAP kinase activity or MAP kinase activity (Fig 2). MAPKK activity was detectable only in cells coexpressing MAPKK and Δ Raf-1. Immunoblot analysis of total cell extracts showed that expression of Δ Raf-1 did not affect the level
15 of expression of MAPKK. The elution pattern of active MAPKK from the Mono Q column was complex with four peaks of activity that correlated well with MAPKK in immunoblots (Fig 2B). In cells expressing Δ Raf-1 the peak of MAPKK immunoreactivity eluted at
20 ca 100mM NaCl (fraction 19) which corresponded to the most active fraction and is similar to the position of the major peak of MAPKK activity from mammalian cells²⁴. In the absence of Raf, most of the MAPKK was found in the column flow-through
25 fractions (Fig 2B). No MAP kinase activity could be detected in any of the extracts.

 The elution pattern of active MAPKK suggested some modification of the protein in cells expressing Raf. Since Raf-1 is a protein kinase we looked at
30 the phosphorylation state of MAPKK in *S. pombe* after metabolic labelling with ³²P-orthophosphate. Although MAPKK was phosphorylated in the absence of Raf-1, coexpression of Raf-1 or Δ Raf-1 led to hyperphosphorylation of MAPKK which was accompanied
35 by a decrease in its mobility upon gel electrophoresis (Figs 2 and 3). We estimated that Raf-1 stimulated MAPKK phosphorylation about 4-fold and

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that Δ Raf-1 gave at least a 5-fold stimulation. In Raf-1 expressing cells the two forms of MAPKK were present in similar amounts while in Δ Raf-1 expressing cells the slower migrating form predominated (Fig 3). Given that Δ Raf-1 is more effective than Raf-1 in activating MAPKK as judged by complementation of byr1 (Table 1) the slower migrating, hyperphosphorylated form of MAPKK is likely to be the biochemically active form.

Phosphoamino acid analysis showed that hyperphosphorylated MAPKK contained phosphoserine and phosphothreonine but no phosphotyrosine (data not shown) in agreement with studies on active MAPKK from *Xenopus* oocytes²⁵.

The hyperphosphorylation of MAPKK in cells expressing Raf could be the result of phosphorylation on new sites, enhanced phosphorylation on the sites phosphorylated in the absence of Raf, or a combination of both mechanisms. To investigate MAPKK phosphorylation in more detail, tryptic phosphopeptide maps of immunoprecipitated MAPKK were generated (Fig 3). The maps from Raf-1 and Δ Raf-1 expressing cells were identical but distinct from the map from cells expressing MAPKK alone (Fig 3). The most heavily labelled phosphopeptide is peptide a in cells expressing Δ Raf-1 but peptide b in cells without Raf. Phosphopeptide c is only seen in cells expressing Raf kinase. MAPKK phosphorylation in the absence of Raf may be the result of autophosphorylation, which is known to occur *in vitro*¹⁴, or to phosphorylation by an endogenous yeast kinase. Whatever the cause, this Raf-independent phosphorylation does not activate the enzyme. MAPKK phosphorylation in the absence of Raf could be the result of autophosphorylation, which is known to occur *in vitro*¹⁴, or to phosphorylation by an endogenous yeast

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kinase. Whatever the cause, this Raf-independent phosphorylation does not activate the enzyme.

Immunoprecipitates of Raf kinase from mammalian cells have been shown to phosphorylate and
5 reactivate phosphatase-treated homogenously pure MAPKK preparations⁷ and bacterially expressed v-Raf can also reactivate partially purified MAPKK⁸; but these experiments do not rule out an intermediate between Raf and MAPKK²⁶. However, the inability of
10 *S. pombe* to activate MAPKK unless Raf is expressed, strongly suggests that Raf directly phosphorylates and activates MAPKK. We observe that coexpression of Raf and MAPKK, but not Raf alone, also suppresses the mating defect of *byr2* (Table 1) which encodes a
15 kinase thought to function upstream of Byr1^{27,28}. The inability of Raf alone to suppress a mutant that contains an intact *byr1* gene shows that Raf cannot activate Byr1. This provides a strong genetic argument that Raf directly phosphorylates and
20 activates MAPKK since a putative intermediate would have to be able to activate mammalian MAPKK but not Byr1, its *S. pombe* homologue.

EXAMPLE 2

25 We coexpressed Mos and MAPKK in a *S. pombe* strain deficient in either *byr1* or *byr2* and found that the mating deficiency of the strains was rescued (Figure 4). Expression of Mos itself had no effect on the mating ability of the *S. pombe* mutants.

30 Coexpression of Mos and MAPKK did not, however, restore mating to a strain defective in *spk1*, the *S. pombe* MAP kinase homologue (Table 2). These results with Mos and MAPKK are essentially identical to the findings with Raf-1 and MAPKK and strongly
35 support the idea that Mos can directly activate MAPKK *in vivo*. To confirm that MAPKK was being activated in the presence of Mos we prepared cell

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extracts from *byr1* mutant strains expressing the mammalian kinases and assayed them for MAPKK activity. The result shows that MAPKK is indeed activated when Mos is coexpressed (Figure 5).

5 Hyperphosphorylation of MAPKK when coexpressed with Mos was indicated by a decreased mobility of MAPKK in SDS-PAGE (data not shown). The ability of Mos to function as a MAPKKK when expressed in *S.pombe* contrasts with the inability of the MBP-Mos fusion
10 protein purified from bacteria to activate MAPKK unless added to a mammalian cell extract (Posada et al., 1993). It seems likely that there is an endogenous component that can activate Mos kinase activity: the identity of the Mos activator(s) in
15 mammalian cells and *S.pombe* is not known.

EXAMPLE 3

Isolation of MAP kinase phosphatase encoding genes

20 The human gene CL100 (3) and its murine homologue 3CH134 (42) have been shown to encode polypeptides that have both serine/threonine and tyrosine phosphatase activity (5,6). When expressed
25 in vitro, the gene product has been shown to be very specific for MAP kinase and leads to its inactivation. Coexpression of the murine gene 3CH134 and the erk2 MAP kinase isoform in mammalian cells leads to the dephosphorylation and
30 inactivation of the MAP kinase (7).

To identify related protein amino acids sequences human CL100 and its murine homologue 3CH134 and the human PAC-1 gene (42), a related T cell specific gene of unknown function, were
35 compared. It proved possible to design degenerate PCR primers, based on conserved regions of the proteins. These primers were used to amplify

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related sequences from cDNA made from poly(A)⁺RNA isolated from the human squamous cell line A431. A fragment of 270bp was purified and subcloned. Of fifty individual clones sequences six proved to be identical to CL100. A further twelve clones were found to be homologous to, but distinguishable from, CL100:- STY2 isolated six times and STY3 four times, with single isolates of STY4 and STY5. In order to identify further related genes, we screened human brain and liver cDNA libraries with a mixed probe from STY2 and -3 PCR products. Several hybridising clones were analysed in more detail by restriction endonuclease mapping and partial DNA sequencing. This resulted in the identification of several additional gene families, STY6-10, with STY1 being CL100. In total nine new genes were identified and these are compared to amino acid sequences of CL100, see Figure 7. The high degree of similarity of these genes suggested that they encode proteins with MAP kinase phosphatase activity.

Cell Culture and RNA Preparation

A431 cells were grown in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Total cellular RNA was prepared with RNazolB (Promega) and poly(A)⁺RNA isolated with Dynabeads oligo(dT)25 (DynaI).

Isolation of CL100-related cDNAs

Two degenerate oligonucleotides TA(T,C)GA(T,C)CA(A,G)GG(A,G,T)GG(T,C,G,A)CC(A,T)GT(A,G,T)GA and AT(G,C,T)CC(A,T)GC(T,C)TG(A,G)CA(A,G)TG(T,C,G,A)AC were designed based on amino acid sequences, YDQGGPVE and VHCQAGI conserved between human and mouse CL100 and the human PAC-1 gene. A431 poly(A)⁺RNA (1µg) was reverse transcribed with SuperScript reverse transcriptase (BRL-GIBCO) and

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subject to PCR on a Techne PHC-1 thermal cyclor with these oligonucleotides (Ashworth, 1993) under the following conditions : 94°C for 30sec, 50°C, 30sec, 72°C 1 min. A 270bp band was purified by agarose gel electrophoresis and subcloned into pBluescript.

Fifty individual subclones were sequenced and of these six proved to be CL100. Twelve others were found to be homologous to but not identical to CL100, and these were grouped as four different potential phosphatases, designated STY2-STY5 with CL100 being STY1.

Structural Analysis of STY cDNAs

One of the cDNA clones isolated from the human brain is full length. Colinear alignments of the STY genes with CL100 show that amino acids around the highly conserved catalytic domain differ, and two conserved regions between CL100 and cdc25 are also present in STY8. Studies on the genomic structure of 3CH134 reveal that the transcription unit is 2.8kbp long and split into four exons [46]. It will be of interest to elucidate the genomic structure of the STY genes, and determine if their promoter regions contain consensus sequences for transcription factors. Preliminary studies suggest that STY8 has a similar gene structure to 3CH134.

Functional Assays

The human CL100 and its murine counterpart 3CH134 function as immediate-early genes whose transcription is rapidly and transiently induced within minutes, with protein accumulation seen in the first hour upon growth factor stimulation [46,47]. As observed for the expression of several immediate-early genes, the rapid increase in growth factor receptor tyrosine kinase activity and subsequent activation of signalling molecules needs to return to normal levels to avoid abnormal growth. One method for accomplishing this implicates protein

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phosphatases whose expression is induced by external signals, such that they are present in the cell only under certain circumstances.

Evidence indicates that when CL100 and 3CH134
5 are expressed *in vitro* [48-50] or *in vivo* [47], the gene product leads to selective dephosphorylation of p42^{mapk} blocking its activation by serum, oncogenic Ras, or activated Raf, whilst the catalytically inactive mutant of the phosphatase augments MAP
10 kinase phosphorylation.

We tested whether the phosphatase STY8 exhibited similar specificity *in vivo* using a COS cell transient expression system. We cotransfected Cos cells with the reporter plasmid pEXV3-Myc-p42^{mapk}
15 together with various plasmids including pMT-Myc-STY8. Figure 8 is typical of such an experiment.

It can be seen that in the absence of stimulatory ligand (EGF) the anti-myc antibody 9E10 reveals only a single band of MAP kinase on western blotting (lane 1). In the presence of EGF (lane 2)
20 a clear doublet of bands is present indicating the partial phosphorylation of the MAP kinase. This is unaffected by expression of the parental expression vector (lanes 3 and 4). However expression of CL100
25 or STY8 in the presence of EGF (lanes 7-10) leads to abolition of the EGF induced shift indicating that both these molecules encode MAP kinase phosphatases. Lanes 5 and 6 in which the cells are transfected with Myc-tagged STY8 shows that the STY8 protein is
30 indeed expressed.

The provision of the nucleic acid encoding MAP kinase phosphatases enables incorporation into a yeast screen as described, to look for activators and inhibitors of the MAP kinase pathway and
35 investigate the interaction between various components, in particular MAP kinases and MAPK phosphatases.

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EXAMPLE 4Construction and use of a yeast strain for the
identification of inhibitors of the MAP kinase
pathway

5 A *S. pombe* strain is constructed with the *nmt1*
promoter-*raf*-1 cDNA-*nmt1* terminator (*nmt1-raf*)
integrated at the *byr2* locus in the chromosome.
This is done by first cloning the *nmt1-raf* sequences
10 into the coding region of the *ura4*⁺ gene such that
the *ura4* coding sequence is disrupted and non-
functional to give *ura4::nmt1-raf*. This fragment is
then transformed into a *S. pombe* strain carrying
byr2 disrupted by *ura4*⁺ (*byr2::ura4*⁺; JX3, [19] and
15 transformants resistant to 5-fluoroorotic-acid (FOA),
which selects against cells containing the normal
ura4⁺ gene product, are selected. Some of the FOA
resistant colonies will have the *ura4*⁺ gene at the
byr2 locus replaced by homologous recombination with
20 the disrupted *ura4::nmt1-raf* sequences. This strain
now has *nmt1-raf* stably integrated within the
disrupted *byr2* gene (*byr2::nmt1-raf*). A second
strain is constructed with the *nmt1* promoter-MAPKK
cDNA-*nmt1* terminator (*nmt1-MAPKK*) integrated at the
25 *byr1* locus in the chromosome. This is done as
described above for *nmt1-raf* except that the
recipient strain has *byr1::nmt1-MAPKK*. The
byr2::nmt1-raf and *byr1::nmt1-MAPKK* are crossed by
protoplast fusion, the diploid sporulated and a
30 double mutant strain (*nmt1-raf/MAPKK*) carrying both
byr2::nmt1-raf and *byr1::nmt1-MAPKK* is identified by
tetrad analysis.

 A reporter construct consisting of the promoter
sequence of the pheromone-induced gene *matPm* (which
35 is induced by action of the MAPK pathway) upstream
of the *E. coli lacZ* gene encoding β -galactosidase is
then integrated by homologous recombination at the

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leu1 locus in the *nmt1-raf*/MAPKK strain to give the screening strain *nmt1-raf*/MAPKK/*PM-lacZ*. A control strain is produced by coupling a constitutive promoter *adh1* to the *lacZ* gene and integrating this construct by homologous recombination into the yeast genome.

To identify substances that can inhibit the activity of Raf or MAPKK expressed in yeast the *nmt1-raf*/MAPKK/*PM-lacZ* strain and the control strain are exposed or not to the test substance. After a suitable period of time the activity of β -galactosidase in the exposed and non-exposed cultures is determined [51] and compared. Inhibition of β -galactosidase activity in the culture exposed to the substance identifies the substance as a candidate inhibitor of the mammalian protein kinases. The absence of an effect on β -galactosidase activity in the control strain rules out the possibility that the substance is an inhibitor of β -galactosidase.

EXAMPLE 5

Screening for inhibitors or activators of MAPK phosphatases (MKP) expressed in yeast

A yeast strain is constructed as described above that carried *nmt1-raf*, *nmt1-MAPKK*, *nmt1-MAPK*, *nmt1-MKP* and *matPm-lacZ* integrated at the *byr2*, *byr1*, *spk1*, *ade6* and *leu1* loci, respectively. To identify substances that can alter the activity of the MKP the strain is exposed or not to the test substance and β -galactosidase activity is assayed as above. Increased β -galactosidase activity in the culture exposed to the test substance indicates possible inhibition of the MKP or activation of the protein kinases by the substance. Conversely,

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decreased β -galactosidase activity indicates possible activation of the MKP or inhibition of the protein kinases by the test substance.

Summary

5 Intracellular signalling from receptor tyrosine kinases in mammalian cells has been shown to involve the activation of a signal cascade which includes p21^{ras} and the protein kinases p74^{raf-1}, MAP kinase kinase and MAP kinases¹⁻⁸. In the yeasts *S. pombe*
10 and *S. cerevisiae* the response to mating pheromones utilises the Spk1 and KSS1/FUS3 kinases which have sequence homology to vertebrate MAP kinases⁹⁻¹². The recent cloning of cDNAs for mammalian¹³⁻¹⁵ and frog¹⁶ MAP kinase kinases has shown that they are
15 homologous to the *S. pombe* Byr1¹⁷ and *S. cerevisiae* STE7¹⁸ kinases which have been proposed to function upstream of spk1 and KSS1/FUS3 respectively¹⁹⁻²¹. We have demonstrated that mammalian proteins can substitute for components of the yeast pathway.

20 Expression of mammalian MAP kinase kinase alone fails to complement a byr1 mutant of *S. pombe*. When coexpressed with a MAPKK kinase, such as Raf or Mos, however, MAP kinase is activated by phosphorylation and the mating defect of byr1 mutant is rescued.
25 This suggests that the pathways are functionally homologous and shows that the Raf and Mos kinases directly phosphorylate and activate MAP kinase kinase.

30 Yeast which are deficient in byr1 and/or byr2 activity and wherein the deficiency is complemented by coexpression of mammalian MAPKK kinase and MAPKK genes find use in methods of screening for compounds which interfere in one way or another with the MAPK pathway. MAPK phosphatase genes and/or mammalian
35 MAPK can also be introduced into the yeast. Test substances can be screened to identify activators and inhibitors of various components. Activators

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and inhibitors identified in this way are potential therapeutics, useful in the fight against proliferative disorders. The invention provides valuable tools to those working in the field, facilitating the screening of substances and identifying those with potential.

TABLE 1

Cells were grown on sporulation agar (SSA) at 30°C for 4 days and the number of zygotes, asci and unmated cells were counted. Two clones for each transformant were examined and the average mating frequency determined. Numbers in parentheses are the total number of cells counted for each transformant. The full genotypes of the mutant strains are CB53:*h⁹⁰byr1::ura4 Δ RS ade6 leu1 ura4*; CB57:*h⁹⁰ byr2-JM86 ade5 lev1 ura4*. and CB57: *h⁹⁰ spk1::ura4 Δ RS ade6 leu1 ura4*, which was derived from the *spk1* mutant described in ref.9. The plasmids are described in the description of Figure 1, except for the *byr2* plasmid which has the *byr2* gene cloned into pREP42 and the *spk1* plasmid which is from ref. 9. ND, Not determined

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TABLE 1 Mating frequency (%) of <i>byr1</i> , <i>byr2</i> and <i>spk1</i> mutants transformed with mammalian kinase genes			
Plasmids	<i>byr1</i> Δ (CB53)	Mutant Strains <i>byr2</i> (CB59)	<i>spk1</i> Δ (CB57)
MAPKK	0 (1362)	0 (2177)	0 (747)
MAPKK + <i>raf-1</i>	1.36 (736)	ND	0 (854)
MAPKK + Δ <i>raf-1</i>	3.30 (1755)	4.30 (2045)	0 (1050)
<i>raf-1</i>	0 (1574)	ND	ND
Δ <i>raf-1</i>	0 (1538)	0 (27601)	ND
MAPKK + <i>byr2</i>	0 (1708)	ND	ND
<i>byr1</i>	52.0 (477)	ND	ND
<i>byr2</i>	ND	25.1 (742)	ND
<i>spk1</i>	ND	ND	39.0 (267)

TABLE 2 Complementation of *S. pombe* kinase mutants by mammalian Mos and MAPKK

Plasmids	Mutants		
	<i>byr1</i> Δ (CB53)	<i>byr2</i> Δ (CB85)	<i>spk1</i> Δ (CB57)
MAPKK	-	-	-
MAPKK + Mos	+	+	-
Mos	-	-	-
<i>byr1</i> +	++	-	-
<i>byr2</i> +	-	++	-
<i>spk1</i> +	-	-	++

The mating efficiency is shown as ++ (25 to 50%), + (1 to 5%) or - (less than 0.01%). Methods were as described previously (Hughes et al., 1993).

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CLAIMS:

1. A method of screening for a substance which is an inhibitor of mammalian MAPK pathway, which comprises:
taking yeast which is deficient for yeast MAPKK
5 kinase and MAPKK gene activity, and wherein the deficiency is complemented by coexpression of mammalian MAPKK kinase and MAPKK genes;
exposing the yeast to a test substance under conditions which would normally lead to the activation
10 of the yeast MAPK pathway; and
looking for an end point indicative of activation of the yeast MAPK pathway;
whereby inhibition of that endpoint indicates inhibition of the MAPK pathway by the test substance.
- 15 2. A method according to claim 1 wherein the yeast is *Schizosaccharomyces pombe*.
3. A method according to claim 2 wherein the yeast MAPK is Spk1.
4. A method according to claim 1 wherein the yeast is
20 *Saccharomyces cerevisiae*.
5. A method according to any one of claims 1 to 4 wherein the end point is ability of the yeast to mate and/or sporulate.
6. A method according to any one of claims 1 to 4
25 wherein the end point is production of a detectable substance whose production is mediated by the

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activation of MAPK.

7. A method according to claim 6 wherein the end point is expression of a reporter gene leading to a visually detectable signal.

5 8. A method according to claim 7 wherein the expression of the reporter gene gives rise to a coloured product.

9. A method according to any one of the preceding claims wherein a said mammalian gene is a variant of
10 the wild-type gene.

10. A method according to claim 9 wherein the expressed MAPKK Kinase gene is a variant of the wild-type gene.

11. A method according to claim 10 wherein the
15 expressed MAPKK kinase gene is a deletional variant of the wild-type gene.

12. A method according to any one of claims 1 to 11 wherein the mammalian MAPKK kinase is raf.

13. A method according to any one of claims 1 to 11
20 wherein the mammalian MAPKK kinase is mos.

14. A process which comprises, following the identification of a mammalian MAPK pathway inhibitor substance by a method of any one of the preceding claims, the manufacture of that substance.

25 15. A process which comprises, following the identification of a mammalian MAPK pathway inhibitor

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substance by a method of any one of claims 1 to 13, the use of that substance in the preparation of a medicament.

5 16. A process according to claim 15 wherein the medicament is for anti-proliferative treatment of a mammal.

17. A substance identified using a method according to any one of claims 1 to 13 as an inhibitor of mammalian MAPK pathway, for use as a pharmaceutical.

10 18. The use of a substance identified using a method according to any one of claims 1 to 13 as an inhibitor of mammalian MAPK pathway in the manufacture of a medicament for treatment of a proliferative disorder, are inflammatory disorder, a cario-vascular disorder or
15 neurological disease.

19. Yeast which is defective in yeast MAPKK kinase and/or MAPKK gene activity, which defect is complemented by the coexpression of mammalian MAPKK kinase and MAPKK genes.

20 20. Yeast according to claim 19 which is derived from *Schizosaccharomyces pombe*.

21. Yeast according to claim 19 which is derived from *Saccharomyces cerevisiae*.

22. Yeast according to any one of claims 19 to 21
25 containing nucleic acid from which a mammalian MAPK phosphatase is expressible.

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23. Yeast according to any one of claims 19 to 22 wherein mammalian MAPK substitutes for yeast MAPK.

24. A method of screening for a substance which is an inhibitor of mammalian MAPK phosphatase action on MAPK,
5 which comprises:

taking a yeast which is deficient for MAPKK kinase and/or MAPKK gene activity, wherein the deficiency is complemented by coexpression of mammalian MAPKK kinase and MAPKK genes and wherein a mammalian MAPK
10 phosphatase gene is expressible;

exposing the yeast to a test substance under conditions wherein the MAPK phosphatase normally inhibits the yeast MAPK pathway; and looking for an end point indicative of activation of the yeast MAPK
15 pathway;

whereby activation of that endpoint indicates inhibition of MAPK phosphatase action on the MAPK by the test substance.

25. A method of screening for a substance which
20 affects mammalian MAPK phosphatase action on mammalian MAPK pathway which comprises:

taking a yeast which is deficient for MAPKK kinase and/or MAPK gene activity, wherein the deficiency is complemented by coexpression of mammalian MAPKK kinase
25 and MAPKK genes and wherein a mammalian MAPK phosphatase gene is expressible;

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exposing the yeast to a test substance under conditions wherein the MAPK phosphatase is expressed and normally partially inhibits the yeast MAPK pathway; and looking for an end point indicative of activation or further inhibition of the yeast MAPK pathway;

whereby activation of that endpoint indicates inhibition of MAPK phosphatase action by the test substance, and further inhibition of that endpoint indicates either activation of MAPK phosphatase action by the test substance or inhibition of the MAPK pathway by the test substance.

26. A method according to claim 24 or claim 25 wherein mammalian MAPK substitutes for yeast MAPK.

27. A method according to any one of claims 24 to 25 wherein the yeast is *Schizosaccharomyces pombe*.

28. A method according to any one of claims 24 to 25 wherein the yeast is *Saccharomyces cerevisiae*.

29. A method according to any one of claims 24 to 28 wherein the end point is ability of the yeast to mate and/or sporulate.

30. A method according to any one of claims 24 to 28 wherein the end point is production of a detectable substance whose production is mediated by the activation of MAPK.

31. A method according to claim 30 wherein the end point is expression of a reporter gene leading to a

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visually detectable signal.

32. A method according to claim 31 wherein the expression of the reporter gene gives rise to a coloured product.

5 33. A method according to any one of claims 24 to 32 wherein a said mammalian gene is a variant of the wild-type gene.

34. A method according to claim 33 wherein the expressed MAPKK Kinase gene is a variant of the wild-type gene.

10

35. A method according to claim 34 wherein the expressed MAPKK kinase gene is a deletional variant of the wild-type gene.

36. A method according to any one of claims 24 to 35 wherein the mammalian MAPKK kinase is raf.

15

37. A method according to any one of claims 24 to 35 wherein the mammalian MAPKK kinase is mos.

38. A process which comprises, following the identification of a substance which is an inhibitor of mammalian MAPK phosphatase action on MAPK or a substance which affects mammalian MAPK phosphatase action on mammalian MAPK pathway, by a method of any one of claims 24 to 37, the manufacture of that substance.

20

39. A process which comprises, following the identification of a substance which is an inhibitor of

25

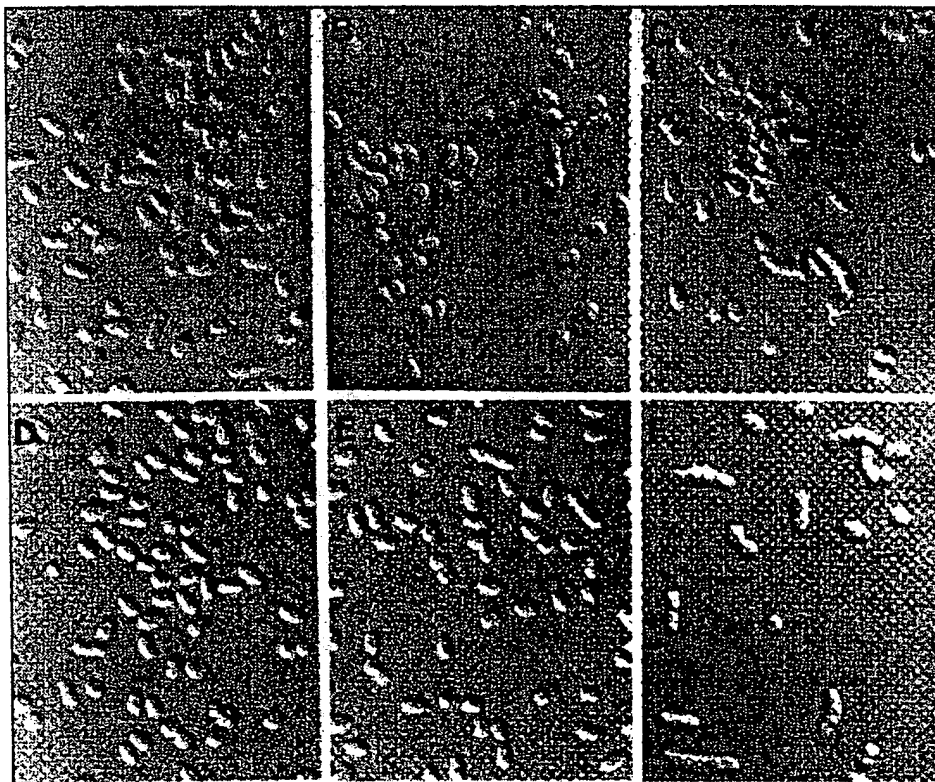
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mammalian MAPK phosphatase action on MAPK or a substance which affects mammalian MAPK phosphatase action on mammalian MAPK pathway, by a method of any one of claims 24 to 37, the use of that substance in
5 the preparation of a medicament.

40. A substance identified using a method according to any one of claims 24 to 37 as an inhibitor of mammalian MAPK phosphatase action on MAPK or as a substance which affects mammalian MAPK phosphatase action on mammalian
10 MAPK pathway, for use as a pharmaceutical.

41. The use of a substance identified using a method according to any one of claims 24 to 37 as an inhibitor of mammalian MAPK phosphatase action on MAPK or as a substance which affects mammalian MAPK phosphatase
15 action on mammalian MAPK pathway, in the manufacture of a medicament for treatment of a proliferative disorder, an inflammatory disorder, a cardio-vascular disorder or neurological disease.

Fig.1.



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Fig.2.

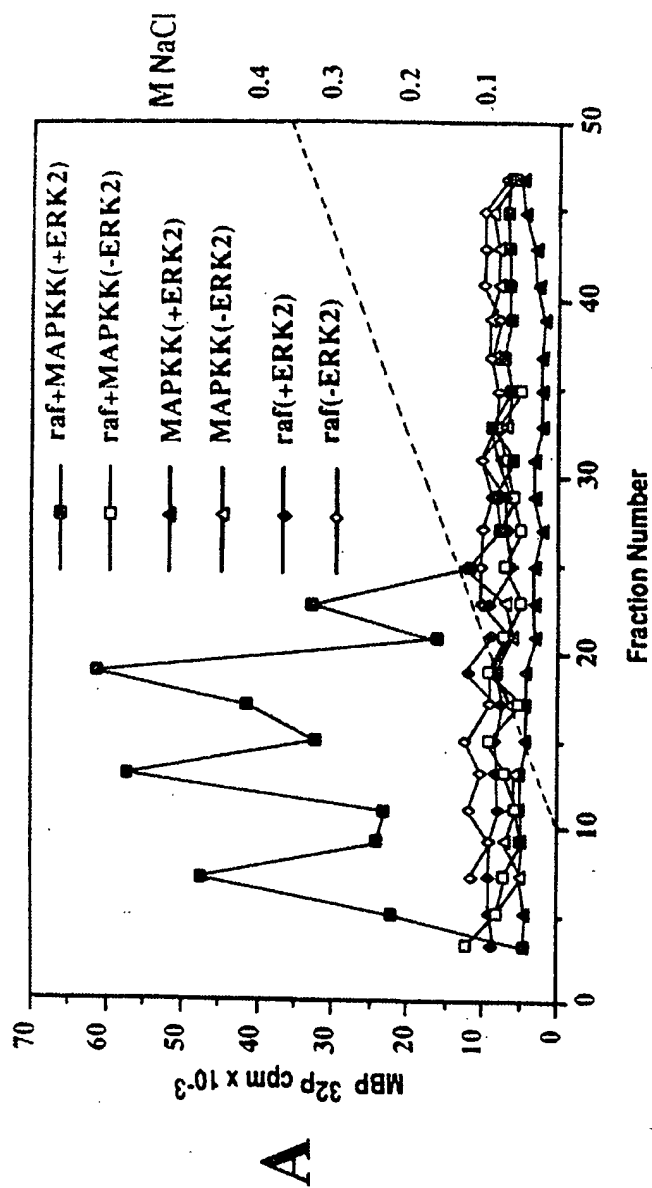
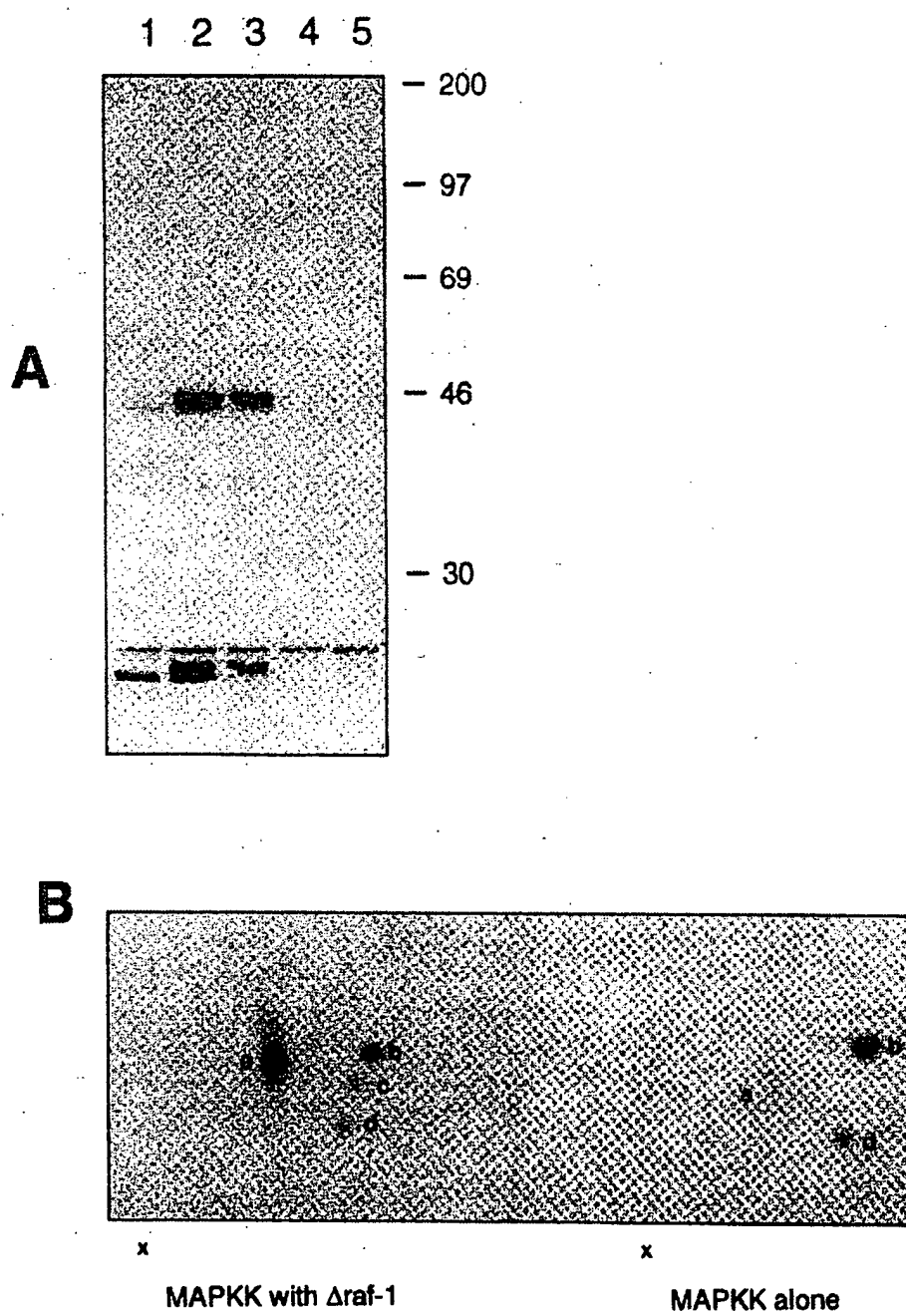


Fig.3.

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Fig.4

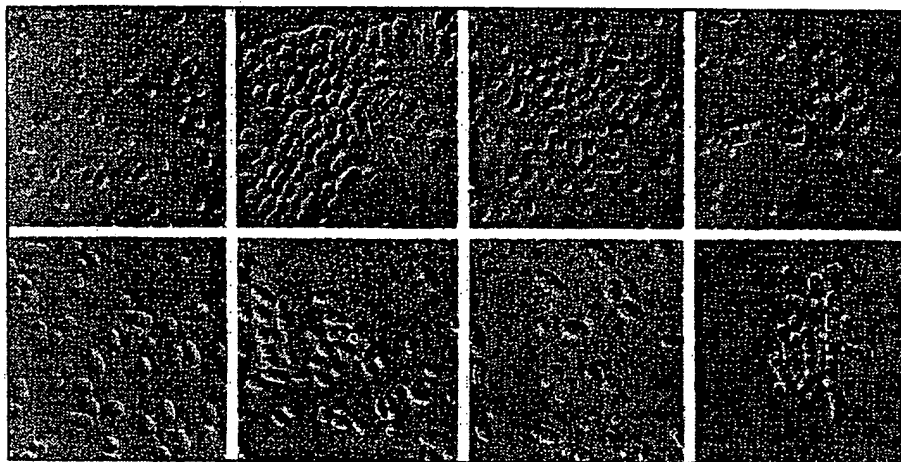
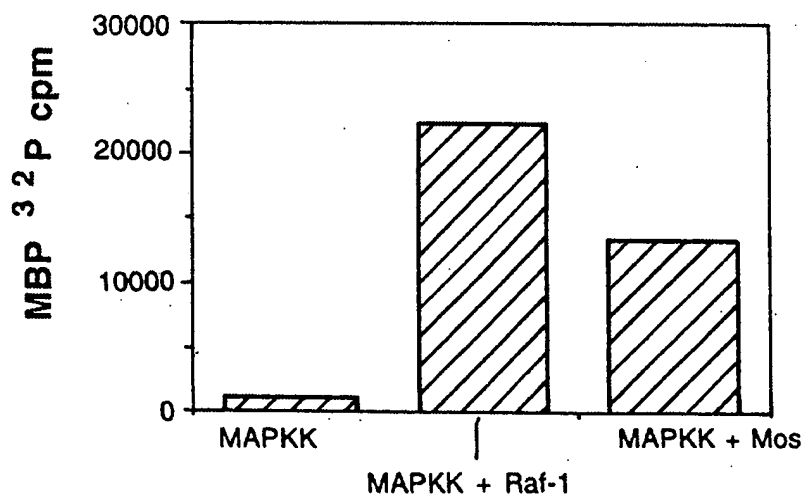


Fig.5.



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Fig.6.

a) STY2

ATC CTT CCC TCC CTC TAC CTT GGA AGT GCC TAC CAT GCA TCC AAG
 TGC GAG TTC CTG GCC AAC TTG CAC ATC ACA GCC CTG CTG AAT GTC
 TCC CGA CGG ACC TCC GAG GCC TGC ATG ACC CAC CTA CAC TAC AAA
 TGG ATC CCT GTG GAA GAC AGC CAC ACG GCT GAC ATT AGC TCC CAC
 TTT CAA GAA GCA ATA GAC TTC ATT GAC TGT GTC AGG GAA AAG GGA
 GGC AAG GTC CTG

b) STY3

ATC CTT CCC TTC CTC TAC CAT GCT AGT GCC TAC CAT GCT GCC CGG
 AGA GAC ATG CTG GAC GCC CTG GGC ATC ACG GCT CTG TTG AAT GTC
 TCC TCG GAC TGC CCA AAC CAC TTT GAA GGA CAC TAT CAG TAC AAG
 TGC ATC CCA GTG GAA GAT AAC CAC AAG GCC GAC ATC AGC TCC TGG
 TTC ATG GAA GCC ATA GAG TAC ATC GAT GCC GTG AAG GAC TGC CGT
 GGG CGC GTG CTG

c) STY4

CCG ATA AGA TTC CTC TAT CTT CTA AAG CTT TAC TCT CCC CGA AAA
 GTC CTC TAC CGC TCC TCC GCC CGG CTC CTC GGT CTG AAG ACA CCG
 AGA CTC GAC CAG ACT CGC CAA CTC

d) STY5

ATC TTG CCC TAC CTG TTC CTG GGC AGC TGC AGT CAC TCG TCA GAC
 CTG CAG GGG CTG CAG GCC TGT GGC ATC ACA GCC GTC CTC AAC GTG
 TCC GCC AGC TGC CCC AAC CAC TTT GAG GGC CTT TTC CGC TAC AAG
 AGT ATC CCT GTG GAG GAC AAC CAG ATG GTG GAG ATC AGT GCC TGG
 TTC CAG GAG GCC ATA GGC TTC ATT GAC TGG GTG AAG AAC AGC GGA
 GGC CGG GTG CTG

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Fig.6(Cont 1)

e) STY6

GCT GAC ATT AGC TCC CAC TTT CAA GAA GCA ATT GAT TTT ATT GAC
 TGC GTC AGG GAA GGA GGA GGC AAG GTC CTA GTC CAC TGT GAG GCT
 GGG GTC TCG AGG TCA CCC ACC ATC TGC ATG GCG TAC CTC ATG AAG
 ACC AAG CAG TTC CGC CTG AAG GAG GCC TTC GAC ATC GTC AAG CAG
 AGG AGG AGC GTG ATC TCT CCC AAC TTT GGC TTT ATG

f) STY7

TCTTGAGAGC TGTGTGGTCG CCATGCTGTC CCCTGAAGCG AGGTGATGCG
 GTACCTGGTC GAAGTGGAGG AGCTGGCCGA GGCGGTGCTG TCGGACAAGC
 GGACGATTGT AGACCTGGAT ACCAAGAGGA AT

g) STY8

CCCGGGTTCT CTTCTCTTCC TCGCGCGCCC AGCCGCCTCG GTTCCCGGCG
 ACCATGGTGA CGATGGAGGA GCTGCGGGAG ATGGA CTGCA GTGTGCTCAA
 AAGGCTGATG AACCGGGACG AGAATGGCGG CGGCGCGGGC GGCAGCGGCA
 GCCACGGCAC CCTGGGGCTG CCGAGCGGCG GCAAGTGCCT GCTGCTGGAC
 TGCAGACCGT TCCTGGCGCA CAGCGCGGGC TACATCCTAG GTTCGGTCAA
 CGTGCGCTGT AACACCATCG TCGCGCGGGC GGCTAAGGGC TCCGTGAGCC
 TGGAGCAGAT CCTGCCCCGCG GAGGAGGAGG TACGCGCCCC CTTGCGCTCC
 GGCCTCTACT CGGCGGT CAT CGTCTACGAC GAGCGCAGCC CGCGCGCCGA
 GAGCCTCCCG GAGGACAGCA CCGTGTCGCT GGTGGTGAG GCGCTGCGCC
 GCAACGCCGA GCGCACCGAC ATCTGCCTGC TCAAAGGCGG CTATGAGAGG
 TTTTCCTCCG AGTACCCAGA ATTCTGTTCT AAAACCAAGG CCCTGGCAGC
 CATCCACCC CCGGTTCCCC CCAGCGCCAC AGAGCCCTTG GACCTGGACT
 GCAGCTCCTG TGGGACCCCA CTACACGACC AGGAGGGTCC TGTGGAGATC
 CTTCCCTTCC TCTACCTCGG CAGTGCCTAC CATGCTGCCC GGAGAGACAT
 GCTGGACGCC CTGGGCATCA CGGCTCTGTT GAATGTCTCC TCGGACTGCC
 CAAACCACTT TGAAGGACAC TATCAGTACA AGTGCATCCC AGTGGAGAT
 AACCACAAGG CCGACATCAG CTCCTGGTTC ATGGAAGCCA TAGAGTACAT
 CGATGCCGTG AAGGACTGCC GTGGGCGCGT GCTGGTGAC TGCCAGGCGG
 GCATCTCGCG GTCGGCCACC ATCTGCCTGG CCTACCTGAT GATGAAGAAA
 CGGGTGAGGC TGGAGGAGGC CTTGAGTTC GTTAAGCAGC GCCGAGCAT
 CATCTCGCCC AACTTCAGCT TCATGGGGCA GCTGCTGAG TTCGAGTCCC
 AGGTGCTGGC CACGTCCTGT GCTGCGGAGG CTGCTAGCCC CTCGGGACCC
 CTGCGGGAGC GGGGCAAGAC CCCC GCCACC CCCACCTCG AGTTCGTCTT
 CAGCTTTCG GTCTCCGTGG GCGTGCACTC GGCCCCCAGC AGCCTGCCCT
 ACCTGCACAG CCCCATCACC ACCTCTCCA GCTGTTAG

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Fig.6 (Cont 2).

h) STY9

ATC CTT GTG GAA GAA GGC CAC ATG GCT GAC ATT AGC TCT CAC TTT
CAA GAA GCA ATA GAC TTC ATT GAC TGT GTC AGA GAA AAG AAA GGC
AAG GTC CTG GTC CAC TGT GAA GCT GGG TTC TCC TGT TCA CCC ACC

i) STY10

AAAGAGTTGT CTACACAGGC ATATATGATA CAGAAGGTGT AGCTCCTACC
AAAAGTGGAG AGCGACAACC CATCCAGATC ACCATGCCGT TCACAGACAT
TGGGACCTTC GAGACAGTGT GGCAAGTCAA GTTCTACAAT TACCACAAGC
GAGACCATTG CCAGTGGGGA AG

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a) Fig.7.

```

CL100  I L P F L Y L G S A Y H A S R K D M L D A L G I T A L I
STY2      L C E F   A N   H           L
STY3      A A R                               L
STY4  P I R       L K L   S P R K V L Y R S S A R L L G   K
STY5      Y   F       C S   S   D L Q G   Q   C           V L

```

```

CL100  N V S A N C P N H F E G H Y Q Y K S I P V E D N H K A D
STY2      R R T S E A C M T   L H       W           S
STY3      S D                               C
STY4  T P R L D Q T R Q L .....
STY5      S           L F R                   Q M V E

```

```

CL100  I S S W F N E A I D F I D S I K N A G G R V F
STY2      H   Q           C V R E K       K   L
STY3      M       E Y   C V   D C R       L
STY4  .....
STY5      A   Q       G       W V   S       L

```

b)

```

CL100  A D I S S W F N E A I D F I D S I K N A G G R V
STY6      H   Q           C V R E G       K

```

```

CL100  F V H C Q A G I S R S A T I C L A Y L M R T N R
STY6  L       E   V       P       M       K   K Q

```

```

CL100  V K L D E A F E F V K Q R R S I I S P N F S F M
STY6  F R   K       D I           V       G

```

c)

```

CL100  I P V E D N H K A D I S S W F N E A I D F I D S
STY9  L       E G   M           H   Q       C

```

```

CL100  I K N A G G R V F V H C Q A G I S R S A T
STY9  V R E K K   K   L       E   F   C   P

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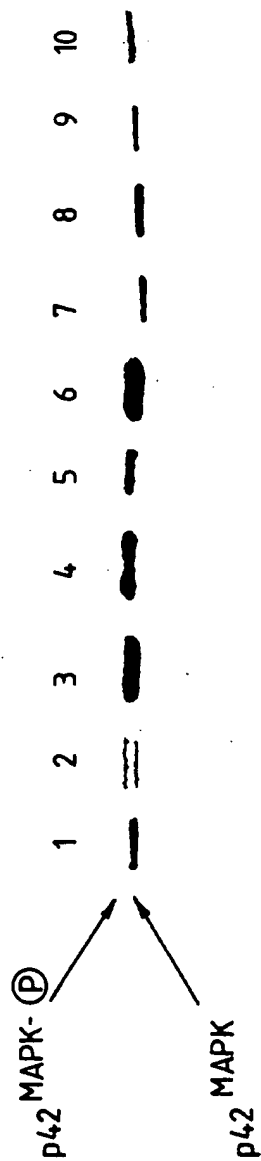
Fig.7 (Cont).

d)

	10	20	30	40	50	60	70	80	90	100
	1234567890123456789012345678901234567890123456789012345678901234567890									
STY8	MYTMEELREMB	CSV	KRMNRDENGCGAGGS	SSHGTGLGLPSGCKCLL	EDCPPEL	AHSAGYILGSVVR	CNTIVRRP	KGSVSL	EOILPAE	EEVRA-R
CL100	MYM	EVGTUBAGGERAIL	-----GER-----	AAQC	HEED	GERSEF	AFNAGH	AGSSVNR	FSII	VRRR
	110	120	130	140	150	160	170	180	190	200
	1234567890123456789012345678901234567890123456789012345678901234567890									
STY8	LXSA	IVYDERS	PRAESL	REDSTV	SEVQAL	RNRUERTD	ICL	IKGGYER	FSSEY	EFCS
CL100	AT	HAVVL	BERSA	ALDGA	KRDG	TLAA	GAAL	CRE	RAAQV	FLKGGY
	210	220	230	240	250	260	270	280	290	300
	1234567890123456789012345678901234567890123456789012345678901234567890									
STY8	PFLEY	GSA	HA	ARRDM	DALG	ITALL	INV	SSDC	PNH	FE
CL100	PFLEY	GSA	HA	ARRDM	DALG	ITALL	INV	SSDC	PNH	FE
	310	320	330	340	350	360	370	380	390	
	1234567890123456789012345678901234567890123456789012345678901234567890									
STY8	VRLE	EAFER	KQRR	STIS	PNF	SENG	QLQ	ESQV	LA	TSCA
CL100	VKLE	EAFER	KQRR	STIS	PNF	SENG	QLQ	ESQV	LA	TSCA

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Fig.8.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00694

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/55 C12Q1/02 C12Q1/68 C12N15/54 C12N1/19
/(C12N1/19, C12R1:645, C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12Q C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EMBO JOURNAL vol. 7, no. 4, 1988, EYNSHAM, OXFORD GB pages 985 - 993 NADIN-DAVIS S. A. ET AL. 'A gene which encodes a predicted protein kinase can restore some functions of the ras gene in fission yeast' see figure 7</p> <p style="text-align: center;">--- -/--</p>	19-21

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

14 July 1994

Date of mailing of the international search report

22.07.94

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Authorized officer

Espen, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00694

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR AND GENERAL GENETICS vol. 235 , 1992 , BERLIN DE pages 122 - 130 STYRKARSDÓTTIR U. ET AL. 'Functional conservation between Schizosaccharomyces pombe ste8 and Saccharomyces cerevisiae STE11 protein kinases in yeast signal transduction' see page 123, left column, last paragraph - page 124, right column, paragraph 1 ---	19-21
X	NATURE vol. 358 , 30 July 1992 , LONDON GB pages 417 - 421 KYRIAKIS J. M. ET AL. 'Raf-1 activates MAP kinase-kinase' see figure 3D ---	17,18
X	ONCOGENE vol. 7 , January 1992 pages 187 - 190 CHARLES C. ET AL. 'cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein' see page 189, right column, last paragraph; figure 1 ---	17,18
P,X	CELL vol. 75 , 5 November 1993 , CAMBRIDGE, MA US pages 487 - 493 SUN H. ET AL. 'MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo' see figures 1-4 ---	17,18
X	TIBS TRENDS IN BIOCHEMICAL SCIENCES vol. 15 , March 1990 , CAMBRIDGE EN pages 98 - 102 COHEN, P. ET AL. 'Okadaic acid: a new probe for the study of cellular regulation' see page 98 see page 102, left column ---	40,41
P,X	NATURE vol. 364 , 22 July 1993 , LONDON GB pages 349 - 352 HUGHES D. A. ET AL. 'Complementation of byr1 in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase' see the whole document -----	1-41